Patent Office
Canberra

I, PETER SCHLAEFER, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PR 3028 for a patent by JOHNSON AND JOHNSON RESEARCH PTY LIMITED as filed on 09 February 2001.

WITNESS my hand this
Fifth day of November 2007

P. Schlaefer

PETER SCHLAEFER
TEAM LEADER EXAMINATION
SUPPORT AND SALES

AUSTRALIA

PATENTS ACT 1990

PROVISIONAL SPECIFICATION

FOR THE INVENTION ENTITLED:-

"GENE SUPPRESSION IN MAMMALIAN CELLS"

The invention is described in the following statement:-

TECHNICAL FIELD

The present invention relates to methods for modifying gene expression and in particular to a method for controlling gene expression in mammalian cells using double-stranded RNAs (dsRNAs), and mammalian cell lines in which gene expression has been altered using the method.

BACKGROUND

The completion of the genomic sequence of a number of different organisms, including humans, is identifying a large number of novel genes for which a biological function is not known. As a consequence, it has become essential to develop effective methods for controlling the expression of specific genes, particularly in mammalian cell culture, for studying the cellular role of these putative genes (Fields, 1997). Functional inactivation of a gene in organisms not amenable to gene disruption has been accomplished using gene constructs expressing sequences that encode antisense RNAs (van der Krol et al., 1988), homologous sense RNAs (Bunnell et al., 1990), ribozymes (Sarver et al., 1990) or dominant negative polypeptides (Herskowitz, 1988). The common feature of all of these forms of *trans*-acting genetic inhibitors is that they are derived from the target sequence. This concept has its origins in the principle of pathogen-derived resistance which suggests that nucleotide sequences derived from a pathogen can be used to genetically modify a host to be resistant to that pathogen (Sanford, 1988). Identification of these gene-specific suppressors is time-consuming and usually requires extensive knowledge of either the domain structure of the protein or screening of large numbers of candidate constructs encoding antisense RNAs, sense RNAs or ribozymes. In most instances this requires experimentation to understand the structural and functional components of the target gene.

More recently, gene-specific double-stranded RNA has been used in some eukaryotic cell types for regulating the expression of specific genes (Fire et al., 1998, Nature 391, 860-811). The most common strategy is the generation of the two complementary RNA strands in vitro, annealing of these strands to form dsRNA, and delivery of this synthesised dsRNA to the target cells. The original studies indicating that dsRNA could regulate specific gene expression showed that this molecule was more effective than either antisense or sense RNA alone and that the mechanism of action of the dsRNA resulted in degradation of the target mRNA. To date, the application of synthetically derived dsRNA to regulate specific gene expression in mammalian cells has been restricted to a single study using non-differentiated mouse oocytes and pre-implantation embryos. Attempts to use this same methodology to inactivate specific genes in cultured mammalian cells has only met with failure. This is most likely due to the fact that differentiated somatic mammalian cells respond to exogenously delivered dsRNA with an interferon response (Marcus, 1983), which includes the activation of a dsRNA-responsive protein kinase (PKR) (Clemens, 1997). This enzyme phosphorylates and inactivates the translation initiation factor EIF2α, resulting in general translational arrest, and eventually cell apoptosis. Thus, *a priori* evidence suggests that the differentiated mammalian cell may be recalcitrant to specific gene inactivation by exogenously-delivered dsRNA.

There is therefore a need for new methods to effectively and predictably alter the expression of a target gene in mammalian cells.

The object of the present invention is to ameliorate at least some of the deficiencies of the prior art or to provide a useful alternative.

The foregoing and following description of, and references to, the prior art is provided so that the present invention may be more fully understood and appreciated in its technical context and its significance more fully appreciated. Unless clearly indicated to the contrary, however, this discussion is not, and should not be interpreted as, an express or implied admission that any of the prior art referred to is widely known or forms part of common general knowledge in the field.

SUMMARY OF THE INVENTION

It has surprisingly and unexpectedly been found that RNA which has the potential to form intramolecular and/or intermolecular dsRNA, can be used to modulate expression of a target gene in mammalian cells.

According to a first aspect, the present invention provides a method of suppressing expression of a target gene or nucleic acid sequence in a mammalian cell including introducing into said cell sense and antisense RNA, with respect to the target gene or nucleic acid sequence, wherein the sense and antisense RNA is capable of forming double-stranded RNA (dsRNA), wherein expression is not suppressed by antisense RNA alone, and with the proviso that said cell is not an oocyte.

Preferably, cells are human cells. It will be clear to the skilled addressee that the cells may be somatic, undifferentiated, dedifferentiated neoplastic, chimera cells or transgenic animal cells. The cells may, of course, be neoplastic cells.

It will also be clear to the person skilled in the art that the cells may be *in vitro* cultured cells or may be *in situ* and that the method has *in vivo* and *ex vivo* therapeutic applications.

The RNA of the present invention may be a single molecule or may be more than one RNA molecule. When the RNA is a single molecule, the dsRNA may be formed by by intramolecular RNA bonding. In embodiments where more than one RNA molecule is used, the dsRNA may be formed by intermolecular RNA bonding.

Preferably, the RNA is encoded by a gene and said gene is transcribed in said cell and more preferably, the gene is delivered to said cell by means of a vector. Most preferably, the vector is a plasmid, adenovirus, adenoassociated virus, or retrovirus. In a preferred embodiment, the plasmid is an episomal plasmid. However, the invention is not limited to these types of vectors and the skilled addressee will be able to identify other suitable vectors.

It will be clear to the skilled addressee in light of the preceeding discussion that any mechanism of introducing RNA which has the potential to form double-stranded RNA into a cell, and particularly into the nucleus of the cell, will be useful in the present invention. As such, it is contemplated, that in certain cases, it may be useful to introduce RNA by, for example, vectors encoding the RNA, microinjection or by vessicle delivery and it will also be clear that the RNA may be in either be in single or double-stranded form at the time of introducing it into the cell. The RNA may, therefore be synthesised outside said cell by standard techniques.

Preferably, the RNA is retained within the nucleus of said cell. In one embodiment, the RNA is retained within the nucleus of said cell by deletion or cleavage

of the polyadenylation signal. Cleavage of the polyadenylation signal from the RNA may be achieved by a *cis*-acting ribozyme or by any other suitable means.

According to a second aspect, the present invention provides a mammalian cell in which a gene or nucleic acid sequence has been suppressed by a method according to the first aspect.

According to a third aspect, the present invention provides a method of modulating expression of a gene or a nucleic acid sequence in mammalian cells including exposing said cells to medium in which mammalian cells according to the second aspect have been grown.

According to a fourth aspect, the present invention provides a method of determining the function of a gene or a nucleic acid sequence including suppressing expression of the gene or nucleic acid sequence by a method according to the first or third aspect.

According to the fifth aspect, the present invention provides a method of determining the function of a protein by suppressing expression of the gene encoding the protein by a method according to the first or third aspect.

According to the sixth aspect, the present invention provides a method of modulating a cellular response wherein said response is due either directly or indirectly to the expression of a gene or nucleic acid sequence and wherein expression of said gene or nucleic acid seqeunce is suppressed by a method according to the first or third aspect.

According to a seventh aspect, the present invention provides a method of treating a disorder resulting either directly or indirectly from expression of a gene or nucleic acid sequence wherein expression of said gene or nucleic acid sequence is suppressed by a method according to the first or third aspect.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Reduction in destabilised green fluorescent protein (dEGFP)-mediated cell fluorescence in human embryonic kidney cells co-expressing sense and antisense dEGFP RNAs.

Figure 1A. Schematic representation of the dEGFP target gene, sense genes and antisense genes used in Example 2. The integrated structure of the dEGFP target gene in the dEGFP-expressing cell line is indicated at the top of the figure. The dEGFP open reading frame (ORF) is under control of the CMV immediate early promoter and the SV40 polyadenylation signal. The sense and antisense dEGFP genes contained on episomal plasmids are indicated with the designation of each expression plasmid indicated at the left. The downward arrow indicates the single base change converting the ATG start codon in the dEGFP ORF to a CTG. The direction of the horizontal arrows indicates the natural direction of transcription. Other abbreviations are as follows: EF-1α, elongation factor 1α promoter; Pur^R, puromycin-N-acetyl transferase; RSV, Rous sarcoma virus long terminal repeat; Hyg^R, hygromycin B phosphotransferase; dEGFP ORF; dEGFP open reading frame. Each of the sense and antisense genes is shown linked with the selectable marker resident on the episome.

Figure 1B. Effect of co-expressing sense and antisense dEGFP RNAs on dEGFP-mediated cell fluorescence. Two to three independent pooled populations for the indicated co-transfected plasmids were assayed for dEGFP-mediated cell fluorescence after growth to three different stages of confluence. The histograms represent the average geometric mean fluorescence and the error bars indicate the standard deviation. The

legend describing each of the co-transfected populations is shown at the top of the diagram. The abbreviations used are as in figure 1A.

Figure 2. dEGFP mRNA steady-state levels in human embryonic kidney cells co-expressing sense and antisense dEGFP RNAs.

Figure 2A. Northern analysis of the steady-state level of dEGFP mRNA. Total RNA isolated from the indicated co-transfected populations was separated on an agarose-formaldehyde gel, transferred to Magna nylon membrane and probed with dEGFP DNA. The signals for the dEGFP mRNA (1.1 kb) and the 18S rRNA (1.8 kb) are indicated.

Figure 2B. Quantitative analysis of the level of dEGFP mRNA relative to the 18S rRNA. The steady-state level of dEGFP target mRNA is expressed as a ratio of 18S rRNA. The legend for the histogram is indicated to the right in the diagram.

Figure 3. dEGFP protein levels in human embryonic kidney cells co-expressing sense and antisense dEGFP RNAs.

Figure 3A. Western analysis of the steady-state level of the dEGFP protein. The signals observed for the dEGFP and β-actin proteins are as indicated.

Figure 3B. Quantitative analysis of the level of dEGFP protein relative to β-actin. Each histogram represents the ratio of the dEGFP protein to the β-actin protein as quantitated using Figure 3A.

Figure 3C. Western analysis of the steady-state level and phosphorylation state of PKR. The position of the 69 kDa PKR monomer is shown. There is no indication of a phosphorylated form.

Figure 4. Suppression of dEGFP-mediated cell fluorescence by dsRNA conditioned medium.

Figure 4A. Overview of the culture medium transfer experiment.

Figure 4B. Suppression of dEGFP-mediated cell fluorescence by dsRNA conditioned medium derived from cells co-expressing sense and antisense dEGFP RNA. The outline of the experiment is summarised in figure 4A. The code for the different histograms is shown at the bottom of the diagram.

Figure 5. Suppression of dEGFP-mediated cell fluorescence by expression of dsRNA from an inverted repeat plasmid.

Figure 5A. Schematic representation of the expression cassettes contained on the inverted repeat plasmids used in Example 4. Each of the three cassettes used to generate dEGFP-specific dsRNA is indicated. All of these inverted repeat genes are under control of the conditional ecdysone-inducible promoter (represented by HSP). The synthetic intervening sequence (IVS) is shown in the first two cassettes. The arrows indicate the normal direction of transcription. Each of these expression cassettes resides on an episomal plasmid containing the RFP gene.

Figure 5B. Effect of expressing dEGFP-specific inverted repeat dsRNAs on dEGFP-mediated cell fluorescence. The level of dEGFP-mediated cell fluorescence relative to the control vector in the plasmid transfected population (RFP+) at 48 hours post-electroporation and 24 hours following the addition of ponasterone A (10 μM). The 'vehicle' referred to in the figure is ethanol alone, while "ponasterone" denotes addition of the inducer ponasterone A.

Figure 6. Effect of a *cis*-acting ribozyme on nuclear localisation of sense RNA.

Figure 6A. Schematic of expression cassettes used in Example 6. Each of the reporter cassettes used to test the efficacy of a *cis*-acting hammerhead ribozyme for localising sense GFP RNA inside the nucleus. The abbreviations are as indicated in figure 1A, with the exception of GFP, which represents green fluorescent protein, and RBZ which represents the sequence encoding the hammerhead ribozyme.

Figure 6B. Effect of a *cis*-acting ribozyme on nuclear localisation of sense RNA. The effect of the constructs indicated in figure 6A on the level of GFP-mediated cell fluorescence. All values are expressed as a percentage of the control cells.

DESCRIPTION OF THE INVENTION

The present invention relates to methods of controlling the expression of specific genes or nucleic acid sequences in mammalian cells using sense and antisense RNA (with respect to the gene or nucleic acid sequence), the expression of which has the potential to form intramolecular and intermolecular dsRNA.

A preferred embodiment of the invention will now be described by way of example only.

EXAMPLE 1. Materials and methods to exemplify the invention

Construction of episomal expression vectors

Standard gene cloning methods were used to construct expression plasmids used in the present study (Sambrook et al., 1989). The plasmids used in the co-transfection experiments were based in the core episomal plasmids pREP7 (Invitrogen) or pEAK10 (Edge Biosystems). These plasmids are maintained within the nucleus and do not generally integrate into the genomic DNA. The sequences required for episomal plasmid maintenance are the Epstein Barr virus OriP and EBNA1 regions. The portion of the dEGFP target gene used to construct the sense and antisense dEGFP-expressing plasmids in pREP7 spanned positions 666 to 1749 in reference to the pd4EGFP-N1 (Clontech) sequence map. This region was PCR-amplified using pd4EGFP-N1 as a template and the following primers: 5' TGA GGA TTC ACC GGT CGC CAC CCT GGT GAG CAA G 3' and 5' TGA GGA TTC ACA AAC CAC AAC TAG AAT GCA GTG 3' (The base change indicated by C was introduced to eliminate the ATG start codon and ensure that sense dEGFP RNA was not translated). The 1080 bp PCR product was digested with *Bam*HI and subcloned into the unique *Bam*HI site in pREP7 downstream of the RSV LTR promoter in the sense and antisense orientations to produce pR7ctgES and pR7ctgEaS, respectively. The dEGFP insert in plasmid pJEAs was obtained by PCR amplifying the entire transcription unit of the dEGFP gene spanning positions 583 to 1749 (in reference to the pd4EGFP-N1 sequence map) using the following PCR primers: 5' TCA GAT CCG CTA GCG CTA CCG GAC 3' and 5' ACA AAC CAC AAC TAG

AAT GCA GTG 3'. This fragment was ligated to *Bam*HI adaptors created by annealing the following single stranded oligonucleotides: 5' TCT CTA GGG ATC CTC AGT CAG TCA GGA TG 3' and 5' CAT CCT GAC TGA CTG AGG ATC CCT AGA GAA TA 3'. The adaptor-ligated fragment was then digested with *Bam*HI and ligated into the unique *Bgl*II site in pEAK10 (JJR) in the antisense orientation relative to the mammalian protein elongation factor 1α promoter to produce pJEAs. For construction of the plasmid pJctgES, the region of the dEGFP gene in pd4EGFP-N1 spanning positions 666 to 1749 was PCR-amplified using the forward primer 5' TGA AGA TCT ACC GGT CGC CAC CCT GGT GAG CAA G 3' and the reverse primer 5' TGA GAA TTC ACA AAC CAC AACTAG AAT GCA GTG 3'. The *Bgl*II-*Eco*RI digested PCR product was directionally cloned in the sense direction downstream of the elongation factor 1α promoter of pEAK10(JJR) to produce pJctgES. The sense and antisense dEGFP genes contained on pR7ctgES, pR7ctgEaS, pJEAs, and pJctgES are indicated in Figure 1A.

The expression cassettes resident on the inverted repeat plasmids are summarised in Figure 5A. The core plasmid was based on pEAK10 (Edge Biosystems). The elongation factor 1α promoter on pEAK10(JJR) was replaced by the heat shock minimal promoter (containing ecdysone/glucocorticoid response elements), the latter of which is conditionally induced in the EcR293 cell line upon addition of the analog ponasterone A. This was accomplished by PCR amplifying the heat shock promoter region using the forward primer 5' TGA ACT AGT TCT CGG CCG CAT ATT AAG TGC 3' and the reverse primer 5' TGA AAG CTT AAG TTT AAA CGC TAG 3' and pIND (Invitrogen) as a template. The PCR product was digested with *Spe*I and *Hind*III and subcloned directionally into pEAK10(JJR) in place of the elongation factor 1α promoter to produce the plasmid pEAK10(JJR)IND. This vector was further modified to include the RFP

gene derived from pDsRed1-N1 (Clontech). This involved digesting pDsRed1-N1 with *Nhe*I and *Age*I, end-filling, and self-ligating to eliminate the multiple cloning site. The RFP cassette was then PCR-amplified from the modified pDsRed1-N1 using the following PCR primers: 5' GCGC ACT AGT CGT ATT ACC GCC ATG CAT TAG 3' and 5' GCGC ACT AGT ACG CCT TAA GAT ACA TTG ATG 3'. The *Spe*I-digested product was cloned into *Spe*I-linearised pEAK10(JJR)IND to produce pEAK10(JJR)INDRFP. This latter vector was the core plasmid used to construct the inverted repeat plasmids.

For construction of the chimeric gene contained on plasmid pEAK10(JJR)INDRFPPAN, the region of the dEGFP gene spanning position 666 to 1527 (in relation to the pd4EGFP-N1 map) was PCR-amplified from pJctgES using the forward primer 5' GCGC AGA TCT ACC GGT CGC CAC CCT GGT GAG 3' and the reverse primer GCGC GAA TTC CAT CTA CAC ATT GAT CCT AG 3'. This 862 bp fragment was digested with *Bgl*II and *Eco*RI and directionally cloned in the sense orientation downstream of the conditional heat shock promoter in pEAK10(JJR)INDRFP. To complete construction of the chimeric gene on pEAK10(JJR)INDRFPPAN, a 350 bp region from the 5' end of the dEGFP (corresponding to positions 666 to 1020 of the pd4EGFP-N1 vector) was PCR-amplified using the primers 5' TGA GAA TTC AGA TCT ACC GGT CGC CAC CCT GGT TGA GCA AG 3' and 5' TGA GAA TTC CTT CAC CTC GGC GCG GGT CTT GTA G 3', and cloned as an *Eco*RI fragment in the antisense orientation downstream of the 862 bp dEGFP fragment to form the inverted repeat cassette.

To construct the intron-containing chimeric genes contained on plasmids pIR(intron)A and pIR(intron)B, a three step cloning protocol was followed. In the first step, the 862 bp region of the dEGFP gene spanning position 666 to 1527 (in relation to the pd4EGFP-N1 map) was PCR-amplified from pJctgES using the forward primer 5' GCGC AGA TCT ACC GGT CGC CAC CCT GGT GAG 3' and the reverse primer GCGC AGA TCT CAT CTA CAC ATT GAT CCT AG 3', and cloned as a *Bgl*II fragment in both orientations downstream of the conditional heat shock promoter in pEAK10(JJR)INDRFP. In the second step, the 296 bp synthetic intervening sequence spanning positions 974 to 1269 of the vector pIRES-Neo (Clontech) was PCR-amplified using the primers GCGC GGT ACC GAA TTA ATT CGC TGT CTG CGA 3' and 5' GCGC GGT ACC CGA CCT GCA CTT GGA CCT GG 3', and cloned as a *Kpn*I fragment in the sense direction downstream of the dEGFP fragment cloned in the first step. The final step in the construction process involved PCR amplification of the 862 bp region of the dEGFP gene spanning position 666 to 1527 (in relation to the pd4EGFP-N1 map) from pJctgES using PCR primers that introduced *Xba*I and *Eco*RI sites to the amplified fragment. These fragments were cloned directionally downstream of the intron sequences to produce the inverted repeat genes on plasmids pIR(intron)A and pIR(intron)B, as summarised in Figure 5A.

The construction of the plasmids to examine the utility of using a *cis*-acting hammerhead ribozyme to restrict transport of RNAs from the nucleus to the cytoplasm was initiated by PCR-amplifying the humanised GFP open reading frame from pGREENLANTERN (Life Technologies) using the 5' primer TGA AAG CTT GCC GCC ACC ATG AGC AAG GGC GAG and the 3' primer TGA AAG CTT TCA CTT GTA CAG CTC GTC CAT GCC. This DNA was then cloned as a HindIII fragment in

the sense direction under control of the elongation factor 1α promoter on pEAK10(JJR) to produce pEAK10gfps. The *cis*-acting ribozyme-encoding DNA was obtained by sythesising and annealing the following complementary oligonucleotides: 5' GAA TTC AAT TCG GCC CTT ATC AGG GCC ATG CAT GTC GCG GCC GCC TCC GCG GCC GCC TGA TGA GTC CGT GAG GAC GAA ACA TGC ATA GGG CCC TGAT 3' and 5' ATC GGG CCC TAT GCA TGT TTC GTC CTC ACG GAC TCA TCA GGC GGC CGC GGA GGC GGC CGC GAC ATG CAT GGC CCT GAT AAG GGC CGA ATT G 3'. This DNA was digested with *Eco*RI and ligated directionally into the pEAK10gfps plasmid following digestion with *Eco*RI and *Eco*RV. The end result was the plasmid pEAK10gfps+RBZ. For each of the plasmids pEAK10gfps and pEAK10gfps+RBZ, derivatives were constructed in which the SV40 polyadenylation signal downstream of the GFP ORF in pEAK10gfps and the *cis*-acting ribozyme sequence in pEAK10gfps+RBZ was deleted. This produced plasmids pEAK10gfps-pA and pEAK10gfps+RBZ-pA, respectively.

Construction of dEGFP-expressing cell line

The derivative cell line expressing the dEGFP target gene was constructed by electroporating EcR293 cells (Invitrogen) with the plasmid pd4EGFP-N1 (Clontech) that had been linearised with AflII. The transfected cell population was selected in the presence of 500 μg/ml G418 and Neo^R clones expanded and screened for dEGFP expression using fluorescence-activated cell sorting (FACs) analyses. The cell line expressing dEGFP under control of the CMV immediate early promoter was shown to contain a single copy of the dEGFP expression cassette.

Cell culture and methods

EcR293 human embryonic kidney cells (Invitrogen) and their derivatives were maintained in DMEM containing 10% fetal calf serum and supplemented with glutamine, streptomycin and penicillin. This cell line expresses a heterodimer of the ecdysone receptor (VgEcR) and the retinoid X receptor (RXR) that binds a hybrid ecdysone response element in the presence of the analog of ecdysone, ponasterone A (No et al., 1996; Saez et al., 2000). FACs analysis for GFP or RFP expression was performed on the Becton Dickinson FACSORT. Total RNA was extracted from cells using the TRIZol Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Northern hybridisation method for target mRNA detection was performed according to Sambrook et al (1989).

To select for cells co-transfected with two different episomal plasmids, a total of $2.5x10^6$ dEGFP-expressing cells were electroporated with 2.5 μg of each of the plasmids. At 48 hours after transfection, cells were exposed to 0.7 μg/ml of puromycin (to select for pEAK10-based plasmids) and 100 μg/ml hygromycin (to select for pREP7-based plasmids). At 28 days after double selection, cells were then exposed to triple selection by including 500 μg/ml of G418. At five weeks post-electroporation, each of the selected populations was characterised for dEGFP-mediated cell fluorescence, dEGFP protein level and steady-state level of dEGFP mRNA.

To test the inverted repeat plasmids in a transient assay, a total of 5 μg of each of the these plasmids and the control vector was independently electroporated into $1x10^6$ dEGFP-expressing cells. At 48 hours post-transfection, each cell population was treated with either 10 μM ponasterone A (induction conditions) or vehicle alone (no induction).

At 24 hours after this treatment, cells were harvested and analysed for dEGFP-mediated cell fluorescence. This involved gating for RFP positive cells (transfected cells only) and determining the dEGFP fluorescence profile within this sub-population.

To examine the effect of the *cis*-acting hammerhead ribozyme on retention of RNA within the nucleus, each of the constructs indicated in figure 6A was introduced into dEGFP-expressing cells by electroporation. At 48 hours after transfection, cells containing the episomal plasmids were selected by adding 1 μg/ml puromycin. Following three weeks of selection, puromycin resistant cells were harvested and assayed for dEGFP-mediated cell fluorescence.

Western blotting procedures

Cell lysates were prepared using RIPA buffer supplemented with protease inhibitors aprotonin (1 μg/ml), leupeptin (10 μg/ml) and DMSF (100 μg/ml). A total of 60μg of total protein was loaded onto pre-cast 10% agarose Tris-HCL gels (BioRad). Proteins were separated by electrophoresis at 200 volts for 1 hour and transferred to PVDF membrane (Millipore) at 80 volts for 60-90 minutes. This membrane was probed with either GFP mouse polyclonal (Clontech), PKR rabbit polyclonal (SantaCruz) or β-actin mouse monoclonal (SantaCruz) antibodies. Secondary antibody detection was performed using either the goat anti-mouse (horseradish peroxidase (HRP)-linked) or the goat anti-rabbit HRP (SantaCruz), followed by visualisation using the luminol/enhancer chemiluminescent substrate (Amersham).

Media transfer experiments

To examine the effect of the culture medium on dEGFP-mediated cell fluorescence, control cells and cells co-expressing antisense and sense dEGFP RNA

were each seeded in three media types: control cell conditioned medium, sense/antisense cell conditioned medium and DMEM medium. After two and five days in each of these media, both control cells and cells co-expressing antisense and sense dEGFP RNA were assayed for cell fluorescence using FACs.

EXAMPLE 2. The effect of sense RNA, antisense RNA and co-expression of sense and antisense RNA on dEGFP gene expression.

A human embryonic kidney cell line stably expressing the dEGFP gene under control of the cytomegalovirus immediate early promoter (and G418 resistant due to the presence of a linked Neo^R gene) was transfected with episomal plasmids that contained either the Hyg^R gene (conferring resistance to hygromycin) or the Pur^R gene (conferring resistance to puromycin) and sense and antisense expression cassettes. The structure of the cassettes used to express antisense complementary to the target mRNA or sense RNA homologous to the target mRNA are indicated in Figure 1A. The ATG start codon in the sense gene was modified to prevent translation of the encoded sense RNA into dEGFP protein. Following co-transfection with the sense and antisense plasmids, cells containing both episomes and the target gene were selected using puromycin, hygromycin and G418. The control cells contained the two base vectors without antisense or sense genes, while the cells containing the antisense plasmid or sense plasmid only were co-transfected with the appropriate base vector containing the second selectable marker. In this way, all cells selected were resistant to puromycin, hygromycin and G418. After selection, all co-transfectants were subcultured, grown to different levels of confluence and analysed by FACs for their cell fluorescence profile. A summary of these results is indicated in Figure 1B.

The results in Figure 1B indicate that cells containing the vectors alone, the antisense plasmid alone or the sense plasmid alone did not display a reduction in dEGFP-mediated cell fluorescence. This outcome was observed using antisense or sense genes controlled by either the mammalian constitutive EF1α promoter or the Rous sarcoma virus LTR. In contrast, cells containing both the antisense and sense plasmids revealed an approximately 40% to 60% reduction in cell fluorescence controlled by the dEGFP target gene. The same trend was observed in cell cultures grown to three different degrees of confluence. These data show that co-expression of antisense and sense RNAs, in the presence of the target mRNA, is more effective at suppressing the cellular phenotype associated with expression of the target gene in human cells than using an antisense or a sense plasmid alone. Thus, introducing two complementary RNAs, with the potential to form intermolecular dsRNA, into human cells can regulate the expression of a specific gene in mammalian cell culture.

We further characterised the above transfectants for the effect of sense and antisense dEGFP RNA co-expression on the dEGFP target mRNA and protein steady-state levels. Northern analysis of total RNA isolated from control cells, antisense cells, sense cells and cells co-expressing sense and antisense RNAs showed that the all of these cell types displayed the same steady-state level of dEGFP target mRNA (Fig 2 A and B). Therefore under these conditions, the reduction in dEGFP-mediated cell fluorescence in the antisense and sense RNA co-expressing cells did not appear to be due to increased turnover of the target mRNA. To examine the impact of co-expressing these complementary RNAs on the level of dEGFP protein, total protein was extracted from the above cells and analysed using dEGFP-specific and β-actin-specific antibodies (Fig 3A and B). Analysis of these protein blots indicated that the level of dEGFP protein was

reduced by 50% in cells co-expressing antisense and sense RNA compared with all of the other cell types (Fig 3B). This result shows that the observed phenotypic change in cell fluorescence in the antisense and sense co-expressing cells was due to the reduction in the steady-state level of the dEGFP protein. In addition, this suppressive effect was specific for the dEGFP protein and did not alter the steady-state level of β-actin.

A common response of somatic mammalian cells to uptake of dsRNA is the activation of the PKR response that results in phosphorylation of PKR, general arrest of translation and eventually apoptosis. To examine whether co-expression of sense and antisense dEGFP RNAs, with the capacity to form dsRNA, affected the level of PKR we examined the steady-state level of this protein in control cells, antisense cells, sense cells and cells co-expressing sense and antisense RNAs (Fig 3C). In all cell types tested, the level of the PKR protein remained unchanged and there was no evidence of a phosphorylated form of PKR. Thus, delivery of the sense and antisense dEGFP plasmids to the same cell resulted in a specific suppressive effect on dEGFP expression and, surprisingly, did not elicit a general cellular response to the presence of dsRNA, as might have been expected in light of the prior art.

EXAMPLE 3. Transferability of the dsRNA-mediated suppression effect to a different population of cells expressing only the target gene.

It has been noted in earlier studies using dsRNA as a mediator of gene inactivation in non-mammalian cells that a proportion of the suppressive effect can be transferred to other cells *in vivo* (Bosher and Labouesse, 2000) or in culture (Caplen et al., 2000). To examine the transferability of the dEGFP-specific dsRNA-mediated suppressive effect, we conducted a culture medium exchange experiment (Fig 4A).

Conditioned media from control cells and cells co-expressing antisense and sense dEGFP RNA was isolated and used to culture cells co-expressing antisense and sense dEGFP RNA and control cells, respectively. The addition of control medium to cells co-expressing antisense and sense dEGFP RNA did not alter the level of suppression of cell fluorescence (Fig 4B). In contrast, control cells cultured in medium isolated from cells co-expressing antisense and sense dEGFP RNA displayed a reduction in dEGFP-mediated cell fluorescence. Thus, the suppressive effect generated within human cells by co-expressing sense and antisense RNA was transferable to cells that had not been previously exposed to either the sense or antisense RNAs.

EXAMPLE 4. The effect of gene constructs expressing intramolecular dsRNA specific for dEGFP on phenotypic expression of the dEGFP target gene.

Gene-specific dsRNA can be generated by either co-expressing two complementary RNA strands (discussed above) or using cassettes expressing RNAs with internal complementarity (referred to as inverted repeat plasmids), the latter of which express RNA capable of forming intramolecular dsRNA. A series of dEGFP-specific inverted repeat plasmids were constructed (Fig 5A). Each of these plasmids was independently electroporated into dEGFP-expressing human cells and transfected cells identified by the RFP marker contained on the inverted repeat plasmids. This population was then assessed for the effect of inducing expression of the inverted repeat dsRNAs on dEGFP-mediated cell fluorescence (Fig 5B). This analysis showed that at 48 hours post-electroporation (and 24 hours after the addition of ponasterone A to induce expression of the inverted repeat dsRNA), expression of the intramolecular dsRNA containing an internal intron reduced dEGFP-mediated cell fluorescence by ~30% to 50%. This

suppressive effect was more marked upon induction of expression of the conditional promoter controlling the inverted repeat cassettes. The appropriate length of the required inverted repeats can be determined by simple experimentation.

These results indicate that expression of inverted repeat dsRNAs can suppress phenotypic expression of a specific gene in human cultured cells.

EXAMPLE 5. Restricting the expression of dsRNA to the nucleus using a *cis*-acting ribozyme.

One of the proposed limitations to using dsRNA to regulate gene expression in mammalian cells in the presence of a global response mechanism involving minimally PKR induction (Sharp, 1999). The result of activation of these activities is inhibition of cell growth and apoptosis. This general response is proposed to be restricted to the cell cytoplasm. It may be that some of the sense/antisense dsRNA in the experiments described herein is exported from the nucleus to the cytoplasm. As such, we have designed a strategy for avoiding the dsRNA-induced global response that involves expression of dsRNA in the nucleus. To this end, we introduced a DNA sequence encoding a *cis*-acting hammerhead ribozyme into pEAK(JJR)gfps between the GFP ORF and the poly A signal. The *cis*-acting ribozyme will prevent polyadenylation and therefore block migration of the encoded transcript (dsRNA) to the cytoplasm (Liu et al., 1994). To test this concept, 293 cells were transfected with pEAK10(JJR)gfps, with or without the ribozyme, and fluorescence measured at 48 hrs and three weeks post-transfection.

The addition of the *cis*-acting ribozyme to the 3' UTR of the GFP gene reduced fluorescence by 15%. Deletion of the polyadenylation (poly A) module from this construct resulted in cells showing 82% less fluorescence. One possible reason for this reduced expression of the GFP gene may have been the loss of the poly A tail and therefore transcript instability. However, a plasmid lacking both the *cis* ribozyme and the poly A sequences still expressed 98% cell fluorescence. The result obtained with the *cis*-acting ribozyme sequence, in the absence of the poly A signal, suggested the possibility that this construct would be more effective at retaining the encoded RNA (dsRNA) within the nucleus. It would be clear to one skilled in the art that this strategy, and the expression vectors described, could be used to retain two complementary RNAs and inverted repeat dsRNAs within the eukaryotic cell nucleus and thus further reduce the risk of induction of the PKR response.

Without being bound by theory, it is proposed that the mechanism of action of the present invention may be dependent on the actual formation of dsRNA. Further it is proposed that the dsRNA, once formed may be degraded to small fragments and that these fragments may interfere with translation from the mRNA of the target gene or nucleic acid sequence. However, it is of course possible that the mechanism of action is by other means which may or may not include one or more of these steps.

Although the invention has been described with reference to specific examples, it will be clear to those skilled in the art that the invention may be embodied in many other forms.

REFERENCES

Bosher, J.M., Labouesse, M., 2000. RNA interference: genetic wand and genetic watchdog. Nature Cell Biol. 2, E31-E36.

Bunnell, B.A., Fillmore, H., Gregory, P., Kidd, V.J., 1990. A dominant negative mutation created by ectopic expression of an AU-rich 3' untranslated region. Somatic Cell Mol. Genet. 16, 151-162.

Caplen, N.J., Fleenor, J., Fire, A., Morgan, R.A., 2000. DsRNA-mediated gene silencing in cultured *Drosophila* cells: a tissue culture model for the analysis of RNA interference. Gene 252, 95-105.

Clemens, M.J., 1997. PKR- a protein kinase regulated by double-stranded RNA. Int. J. Biochem. Cell. Biol. 29, 945-949.

Fields, S., 1997. The future is function. Nature Genet. 15, 325-327.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific interference by double-stranded RNA *in Caenorhabditis elegans*. Nature 391, 806-811.

Herskowitz, I., 1987. Functional inactivation of genes by dominant negative mutations. Nature 329, 219-222.

Liu, Z., Batt, D.B., Carmichael, G.G., 1994. Targeted nuclear antisense RNA mimics natural antisense-induced degradation of polyoma virus early RNA. Proc. Natl. Acad. Sci. USA 91, 4258-4262.

Marcus, P.I., 1983. Interferon induction by viruses: one molecule of double-stranded RNA as the threshold for interferon induction. Interferon 5, 115-180.

No, D., Yao, T., Evans, R.M., 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc. Natl. Acad. Sci. USA 93, 3346-3351.

Saez, E., Nelson, M.C., Eshelman, B., Banayo, E., Koder, A., Cho, G.J., Evans, R.M., 2000. Identification of ligands and coligands for the ecdysone-regulated gene switch. Proc. Natl. Acad. Sci. USA 97, 14512-14517.

Sanford, J.C., 1988. Applying the PDR principle to AIDS. J. Theor. Biol. 130, 469-480.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Sharp, P.A., 1999. RNAi and double-stranded RNA. Genes Develop. 13, 139-141.

Van der Krol, A.R., Mol, J.N., Stuitje, A.R., 1988. Modulation of eukaryotic gene expression by complementary RNA and DNA sequences. Biotechniques 6, 958-976.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of suppressing expression of a target gene or nucleic acid sequence in a mammalian cell including introducing into said cell sense and antisense RNA, with respect to the target gene or nucleic acid sequence, wherein the sense and antisense RNA is capable of forming double-stranded RNA (dsRNA), wherein expression is not suppressed by antisense RNA alone, and with the proviso that said cell is not an oocyte.

2. A method according to claim 1 wherein the cells are human cells.

3. A method according to claim 1 or 2 wherein the cells are somatic cells.

4. A method according to claim 1 or 2 wherein the cells are undifferentiated, dedifferentiated cells and or neoplastic cells.

5. A method according to claim 1 or 2 wherein the cells are chimeric cells.

6. A method according to any one of claims 1 to 5 wherein said cells are *in vitro* cultured cells.

7. A method according to any one of claims 1 to 5 wherein said cells are *in situ*.

8. A method according to any one of claims 1 to 7 wherein said RNA is a single molecule.

9. A method according to any one of claims 1 to 7 wherein said RNA is more than one RNA molecule.

10. A method according to claim 8 wherein said dsRNA is formed by intramolecular RNA bonding.

11. A method according to claim 9 wherein said dsRNA is formed by intermolecular RNA bonding.

12. A method according to any one of claims 1 to 11 wherein said RNA is encoded by a gene and said gene is transcribed in said cell.

13. A method according to claim 12 wherein said gene is delivered to said cell by means of a vector.

14. A method according to claim 13 wherein said vector is a plasmid, adenovirus, adeno-associated virus or retrovirus.

15. A method according to claim 14 wherein the plasmid is an episomal plasmid.

16. A method according to any one of claims 1 to 11 wherein said RNA is synthesised outside said cell.

17. A method according to claim 16 wherein said RNA is introduced into said cell by means of microinjection and/or delivered to the cell in a vessicle.

18. A method according to any one of claims 1 to 17 wherein said RNA is retained within the nucleus of said cell.

19. A method according to claim 18 wherein said RNA is retained within the nucleus of said cell by deletion or cleavage of its polyadenylation signal.

20. A method according to claim 19 wherein said polyadenylation signal is cleaved from said RNA by a *cis*-acting ribozyme.

21. A mammalian cell in which a gene or nucleic acid sequence has been suppressed by a method according to any one of claims 1 to 20.

22. A method of modulating expression of a gene or a nucleic acid sequence in mammalian cells including exposing said cells to medium in which mammalian cells according to claim 21 have been grown.

23. A method of determining the function of a gene or a nucleic acid sequence including suppressing expression of the gene or nucleic acid sequence by a method according any one of claims 1 to 20 or 22.

24. A method of determining the function of a protein by suppressing expression of the gene encoding the protein by a method according to any one of claims 1 to 20 or 22.

25. A method of modulating a cellular response wherein said response is due either directly or indirectly to the expression of a gene or nucleic acid sequence and wherein expression of said gene or nucleic acid sequence is suppressed by a method according to any one of claims 1 to 20 or 22.

26. A method of treating a disorder resulting either directly or indirectly from expression of a gene or nucleic acid sequence wherein expression of said gene or nucleic acid sequence is suppressed by a method according to any one of the claims 1 to 20 or 22.

27. A vector including DNA encoding an RNA molecule capable of forming dsRNA in the nucleus such that said RNA can effect the suppression of a target gene or nucleic acid sequence.

28. A mammalian cell harbouring nucleic acid capable of forming dsRNA wherein at least one strand of said RNA includes at least a sequence corresponding to sense RNA of a target gene or nucleic acid sequence.

DATED this ninth day of February 2001
JOHNSON & JOHNSON RESEARCH PTY LIMITED

Attorney: IVAN A. RAJKOVIC
Fellow Institute of Patent Attorneys of Australia
of BALDWIN SHELSTON WATERS

Fig 1A

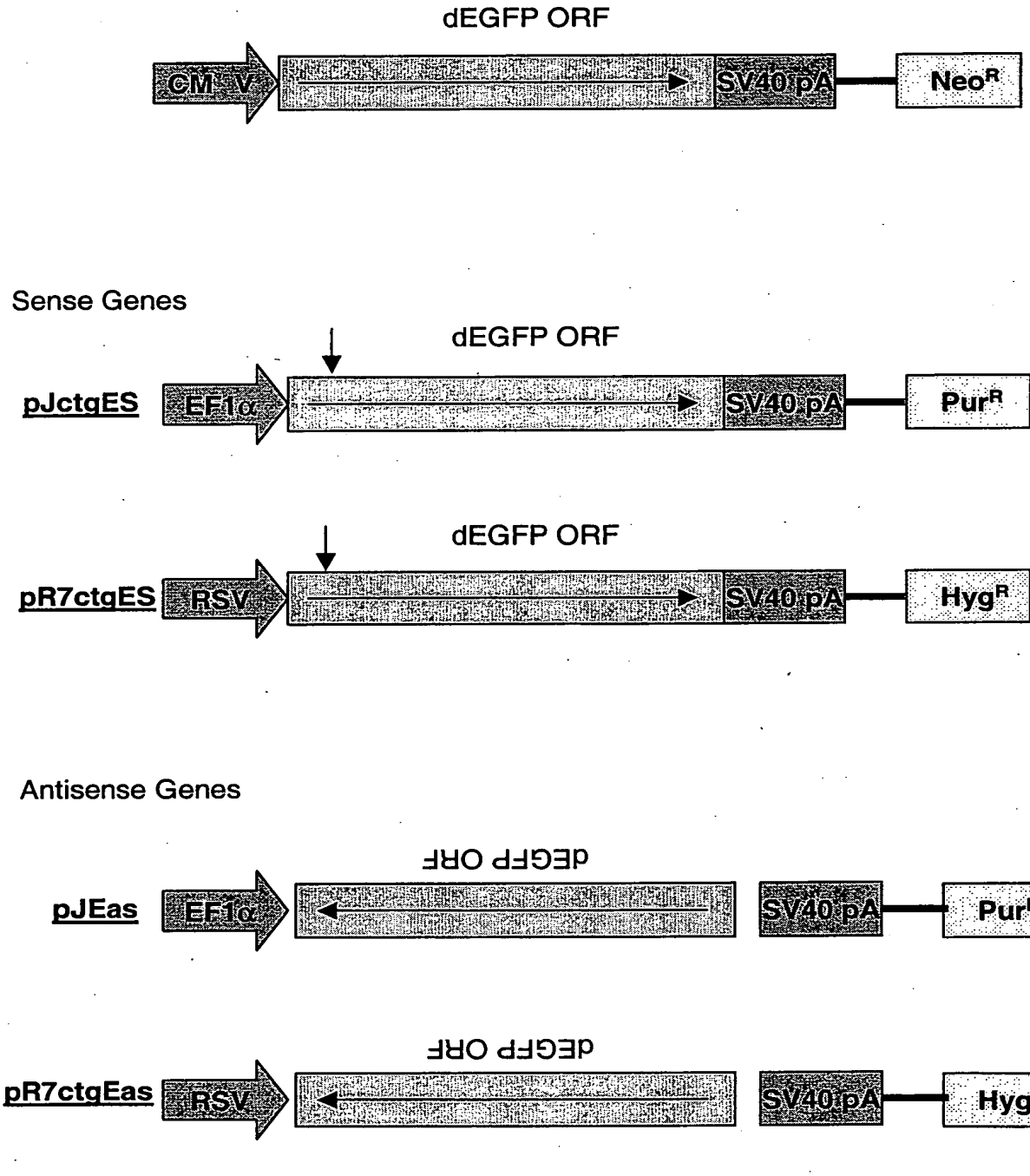

dEGFP ORF
CMV
SV40 pA
Neo^R
Sense Genes
pJctgES
EF1α
dEGFP ORF
SV40 pA
Pur^R
pR7ctgES
RSV
dEGFP ORF
SV40 pA
Hyg^R
Antisense Genes
pJEas
EF1α
dEGFP ORF
SV40 pA
Pur^R
pR7ctgEas
RSV
dEGFP ORF
SV40 pA
Hyg^R

Fig 1B

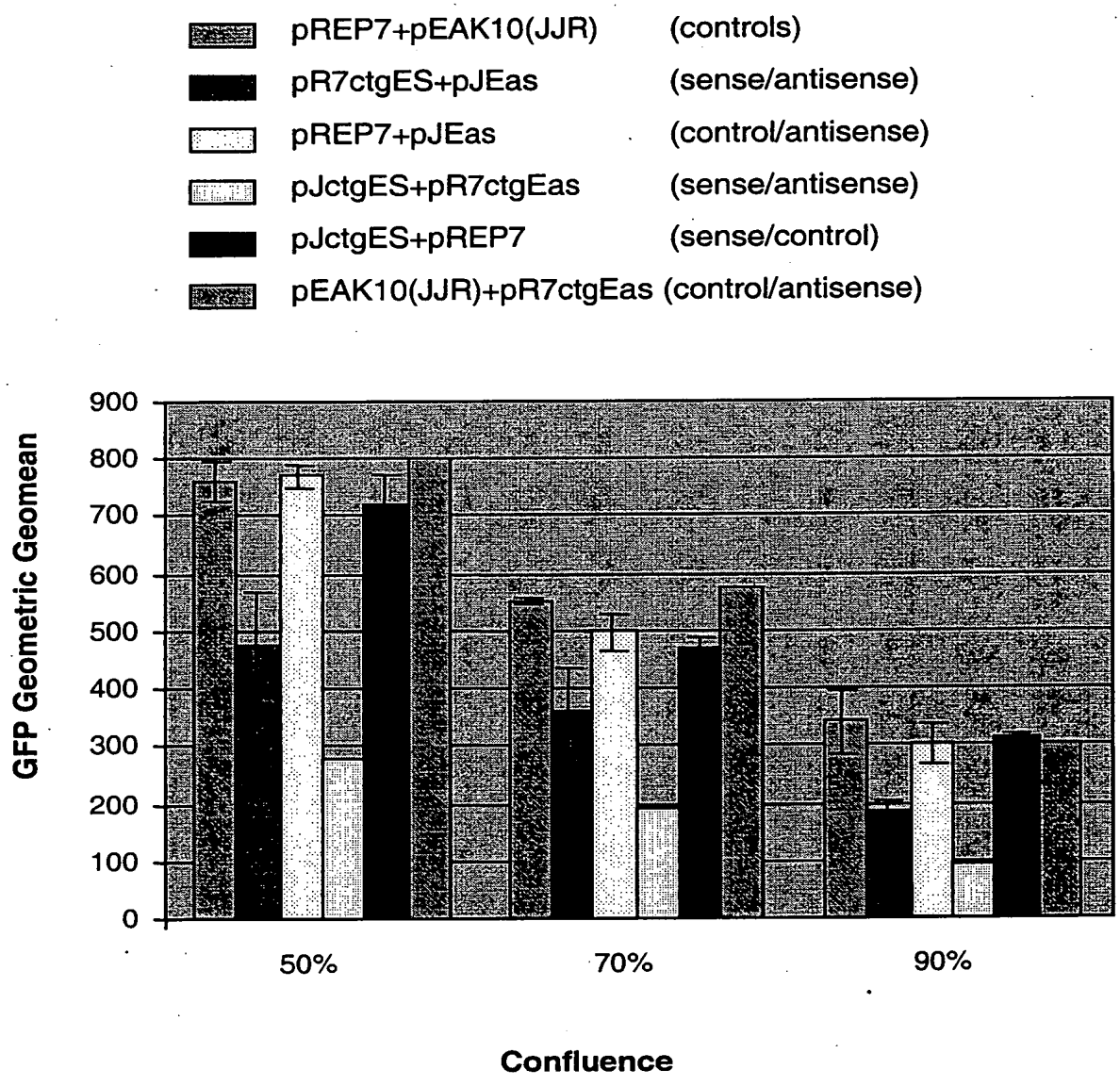

pREP7+pEAK10(JJR) (controls)
pR7ctgES+pJEas (sense/antisense)
pREP7+pJEas (control/antisense)
pJctgES+pR7ctgEas (sense/antisense)
pJctgES+pREP7 (sense/control)
pEAK10(JJR)+pR7ctgEas (control/antisense)
GFP Geometric Geomean
900
800
700
600
500
400
300
200
100
0
50%
70%
90%
Confluence

Fig 2A

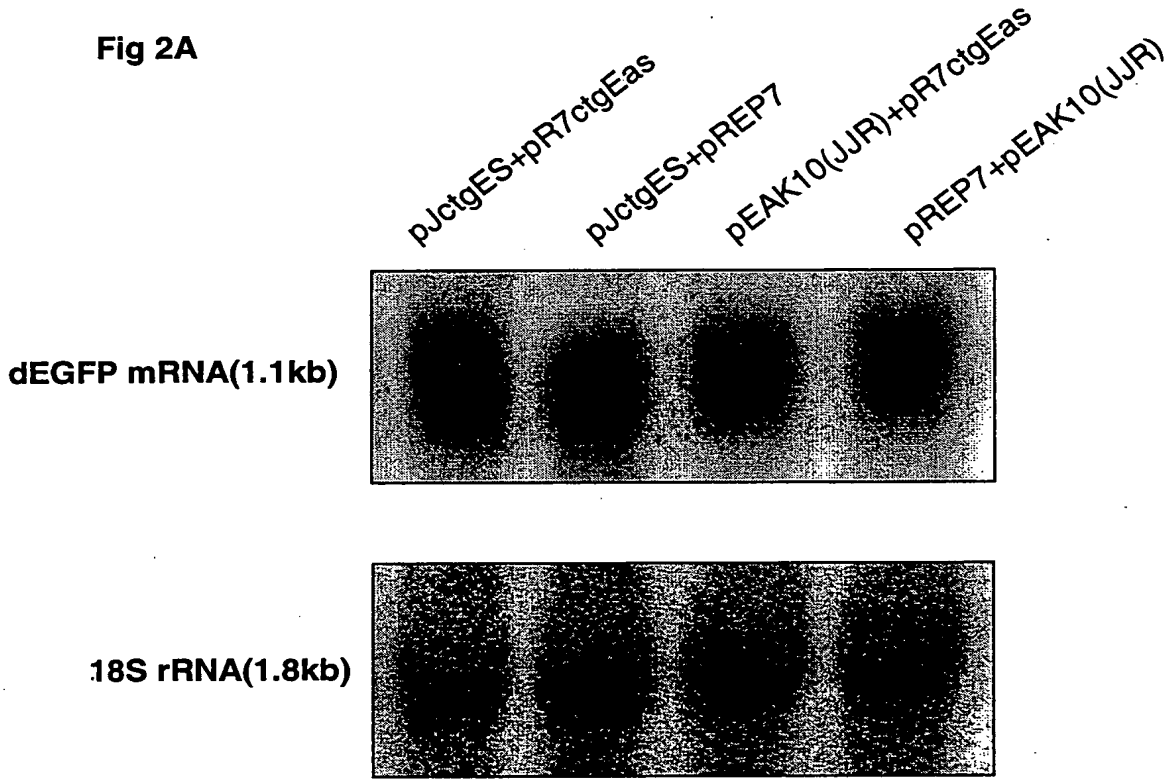

pJctgES+pR7ctgEas
pJctgES+pREP7
pEAK10(JJR)+pR7ctgEas
pREP7+pEAK10(JJR)
dEGFP mRNA(1.1kb)
18S rRNA(1.8kb)

Fig 2B

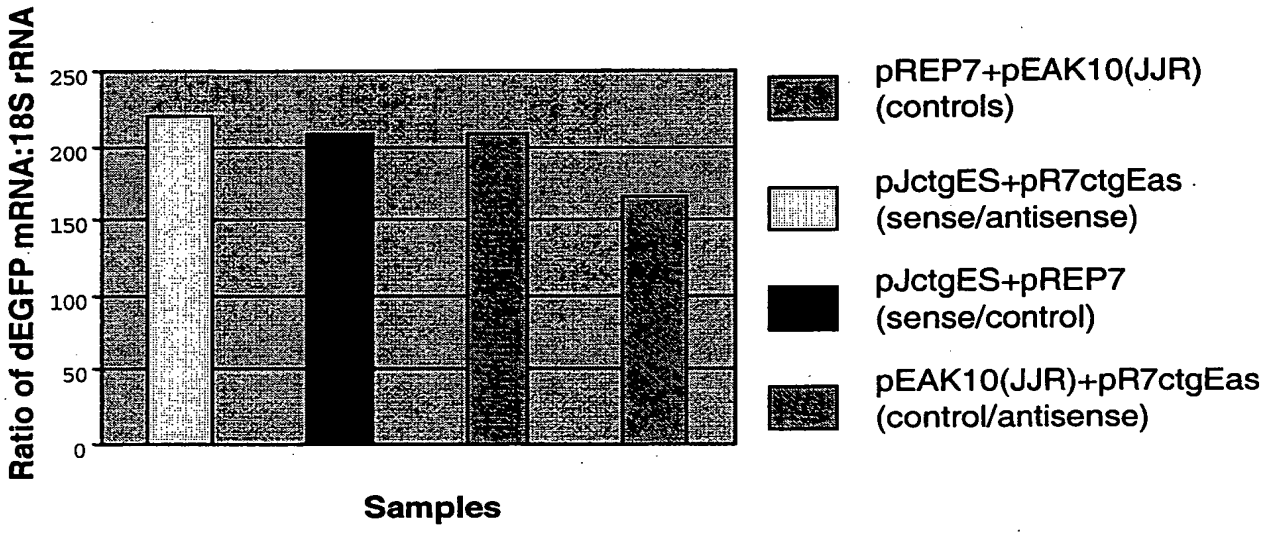

Ratio of dEGFP mRNA:18S rRNA
250
200
150
100
50
0
Samples
pREP7+pEAK10(JJR)
(controls)
pJctgES+pR7ctgEas
(sense/antisense)
pJctgES+pREP7
(sense/control)
pEAK10(JJR)+pR7ctgEas
(control/antisense)

Fig 3A

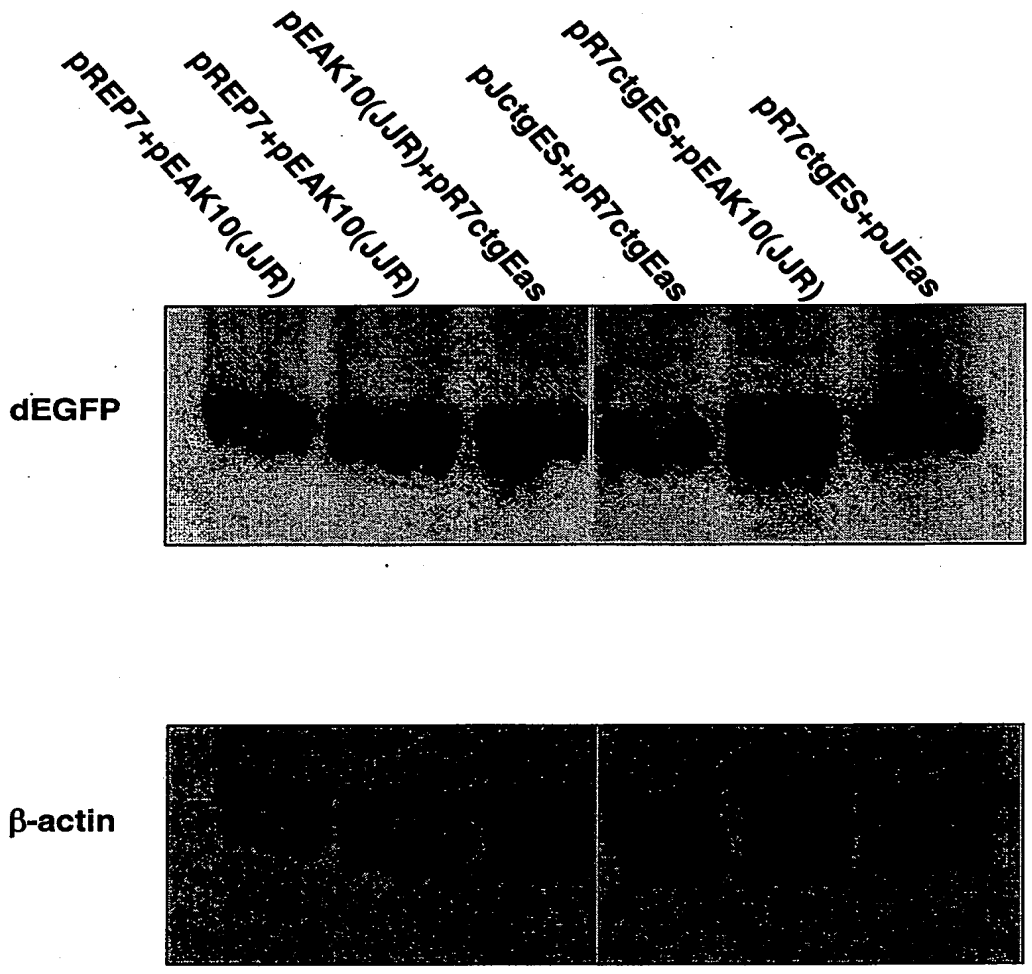

pREP7+pEAK10(JJR)
pREP7+pEAK10(JJR)
pEAK10(JJR)+pR7ctgEas
pJctgES+pR7ctgEas
pR7ctgES+pEAK10(JJR)
pR7ctgES+pJEas
dEGFP
β-actin

Fig 3B

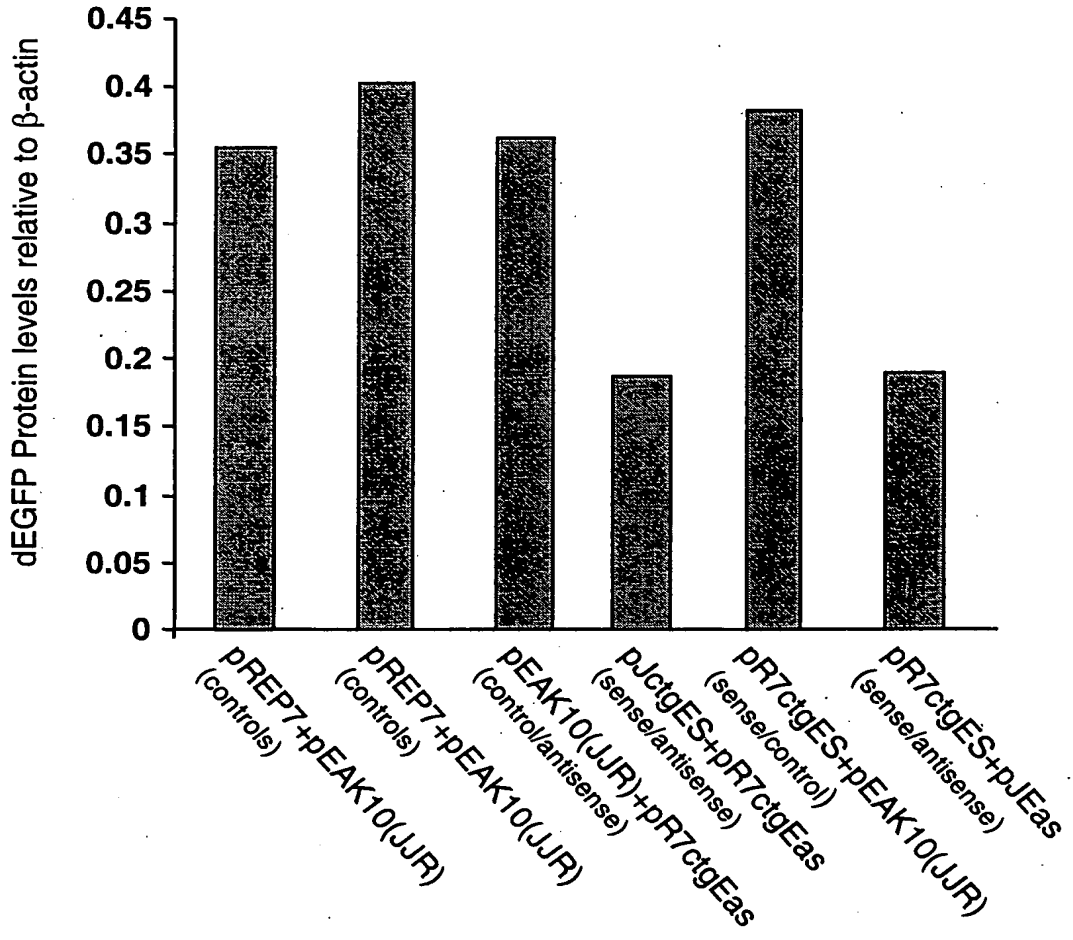

dEGFP Protein levels relative to β-actin
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
pREP7+pEAK10(JJR) (controls)
pREP7+pEAK10(JJR) (controls)
pEAK10(JJR)+pR7ctgEas (control/antisense)
pJctgES+pR7ctgEas (sense/antisense)
pR7ctgES+pEAK10(JJR) (sense/control)
pR7ctgES+pJEas (sense/antisense)

Fig 3C

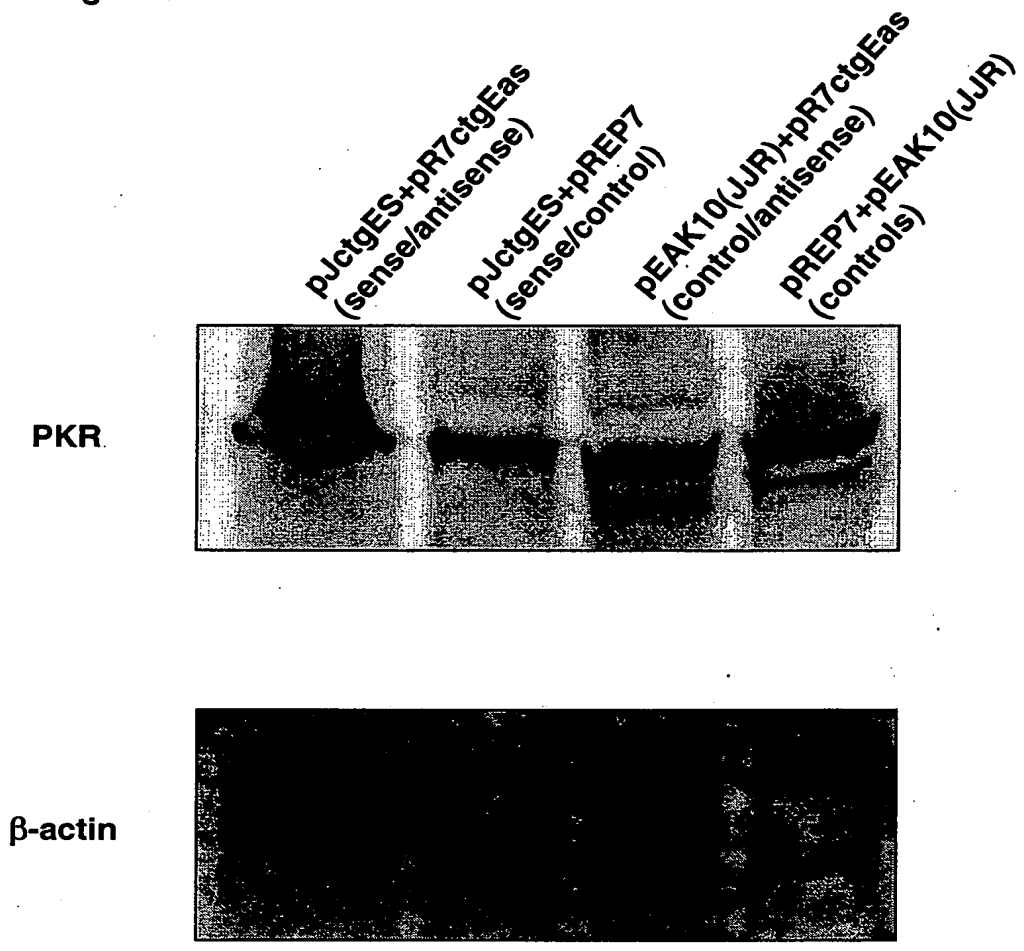

pJctgES+pR7ctgEas (sense/antisense)
pJctgES+pREP7 (sense/control)
pEAK10(JJR)+pR7ctgEas (control/antisense)
pREP7+pEAK10(JJR) (controls)
PKR
β-actin

Fig 4A

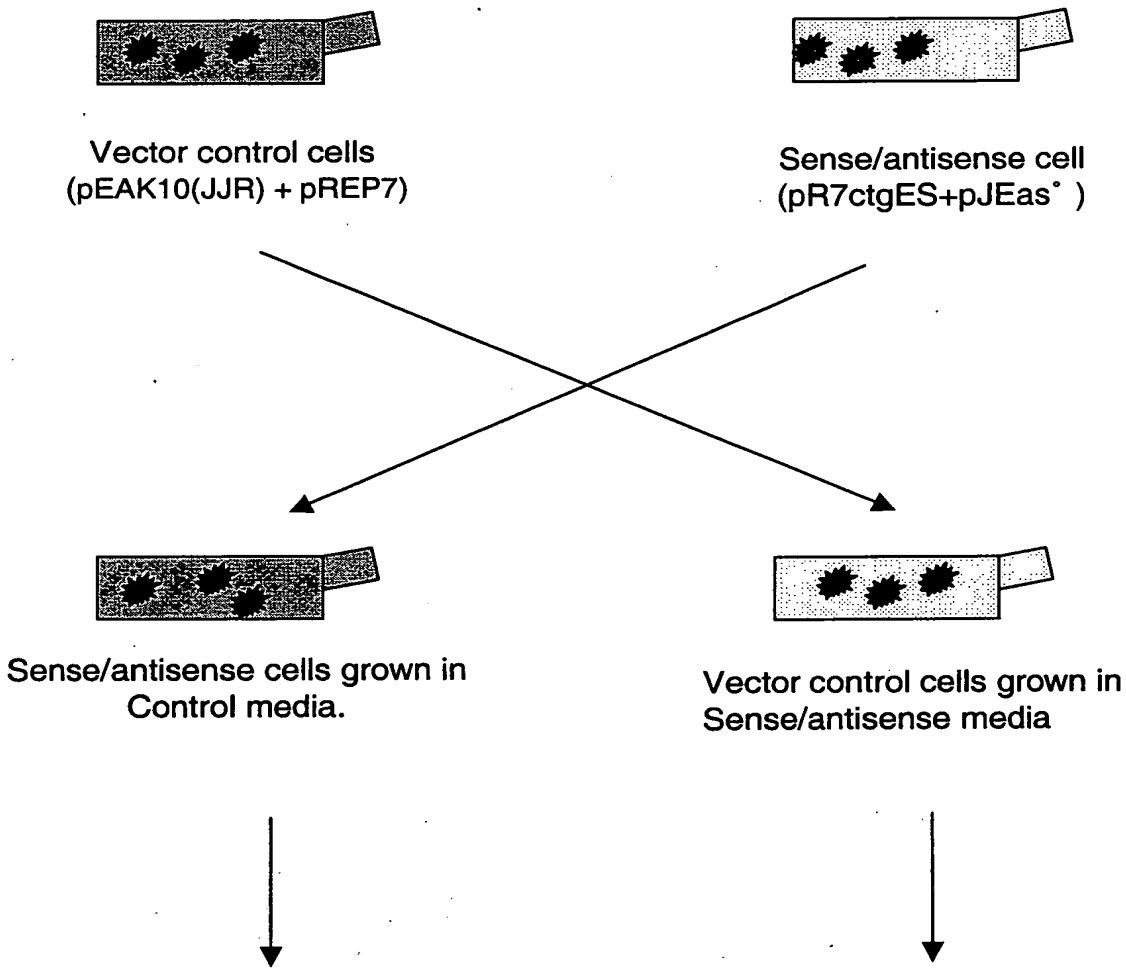
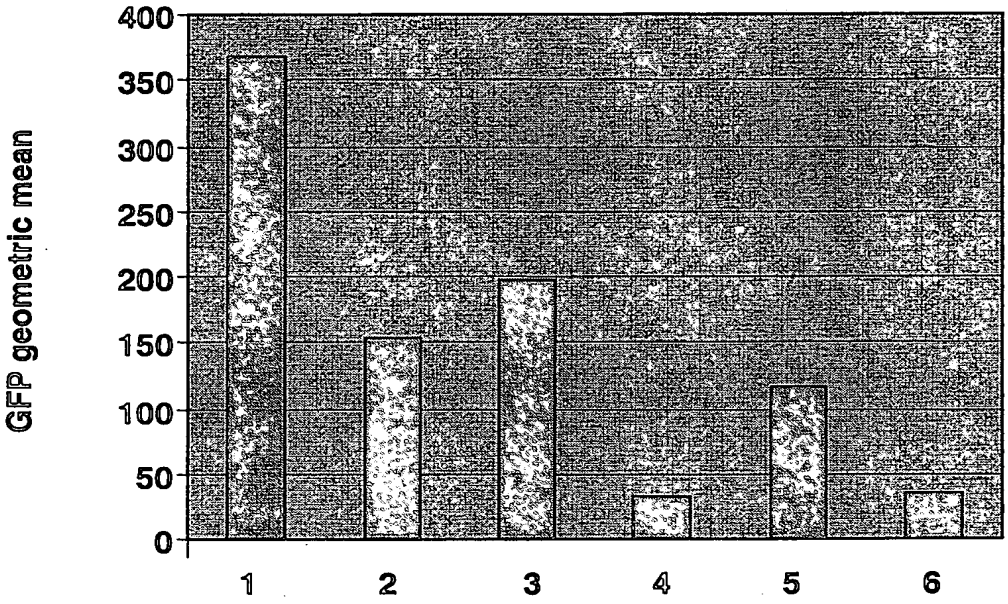

Vector control cells
(pEAK10(JJR) + pREP7)
Sense/antisense cell
(pR7ctgES+pJEas°)
Sense/antisense cells grown in
Control media.
Vector control cells grown in
Sense/antisense media
Incubate cells with indicated media and then
analysed samples using FACS five days after seeding.

Fig 4B



1-pEAK10(JJR) + pREP7 (plasmid controls)

2-pR7ctgES + pJEas (sense/antisense)

3-pEAK10(JJR) + pREP7 (plasmid controls) with DMEM

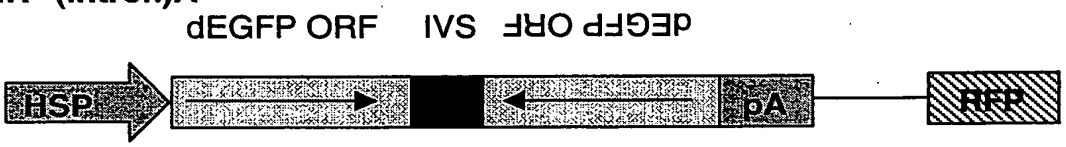
4-pEAK10(JJR) + pREP7 (plasmid controls) with sense/antisense cell medium)

5-pR7ctgES + pJEas (sense/antisense) with DMEM

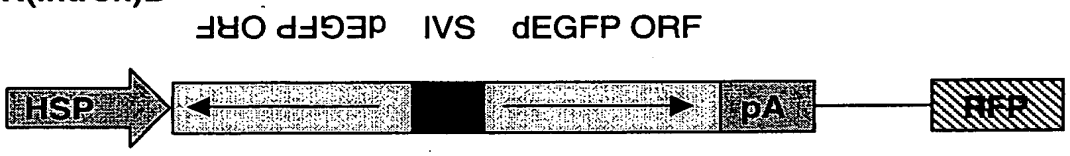
6-pR7ctgES + pJEas (sense/antisense) with sense/antisense cell medium)

Fig 5A

pIR° (intron)A



pIR(intron)B



pEAK10(JJR)INDRFPPAN

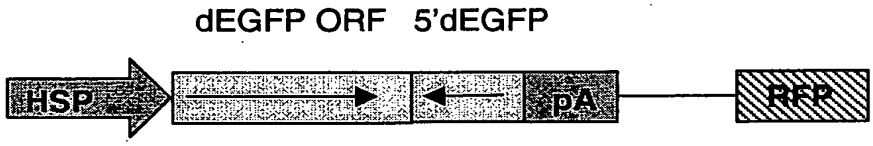


Fig 5B

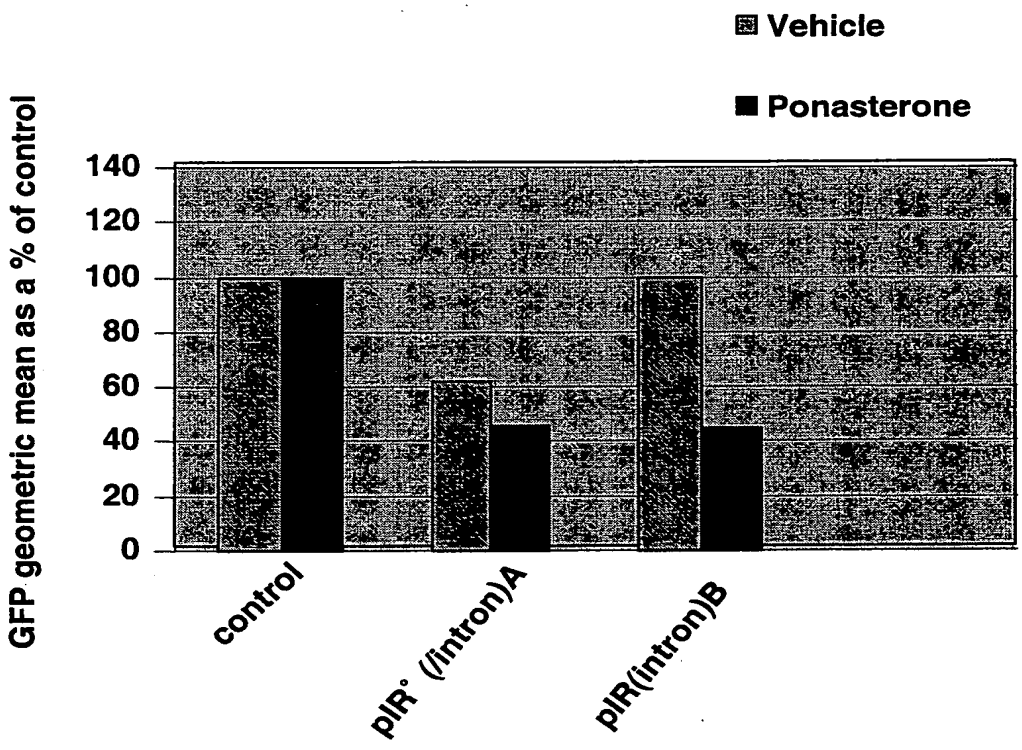

Vehicle
Ponasterone
GFP geometric mean as a % of control
140
120
100
80
60
40
20
0
control
pIR° (/intron)A
pIR(intron)B

Fig 6A

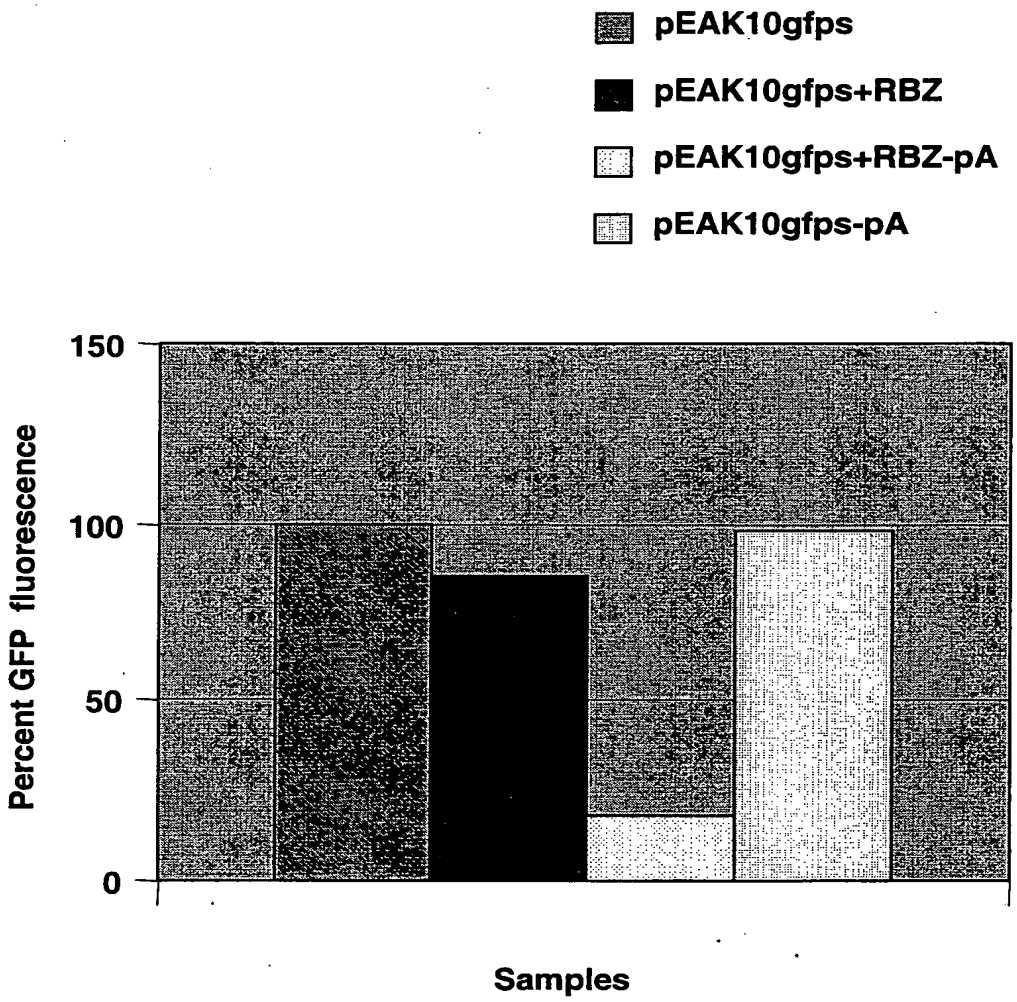
pEAK10gfps

pEAK10gfps+RBZ

pEAK10gfps+RBZ-pA

pEAK10gfps-pA

Fig 6B


pEAK10gfps
pEAK10gfps+RBZ
pEAK10gfps+RBZ-pA
pEAK10gfps-pA
150
100
50
0
Percent GFP fluorescence
Samples